DEVELOPMENT OF A LUMINEX®-BASED MOLECULAR ALTERNATIVE FOR CLASSICAL MICROBIOLOGICAL SUBTYPING TECHNIQUES, SALMONELLA PHAGE TYPING AS A CASE STUDY

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INTRODUCTION

WIV-ISP is responsible for the diagnosis and surveillance of numerous microbial diseases. Therefore, microbial isolates should be characterised at a level suitable for routine surveillance, for outbreak detection and for tracing the source of an infection. In actual practice, this means for most pathogens that a subtyping technique is needed to allow characterisation below the serovar level. The development of molecular alternatives for classical microbiological subtyping methods allows for improvements in standardisation, accuracy and rapidity. The PhD project SalMolType, which is a collaborative effort by the scientific unit Platform of Biotechnology and Molecular Biology and the Bacterial Diseases unit, aims at developing such a molecular alternative subtyping method and takes *Salmonella* phage typing as a case study.

Salmonella is one of the most frequent causes of food-borne outbreaks and human salmonellosis is the second most frequently reported zoonosis in the European Union **(1)**. The most common serovars of *Salmonella* isolated from human outbreaks are *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S*. Enteritidis) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S*. Typhimurium) **(1)**. Typing methods which allow characterisation below the serovar level are essential in a surveillance programme for this diverse genus. Classical surveillance programmes for *Salmonella* rely on phenotyping methods such as phage typing and antimicrobial susceptibility testing. Phage typing is a subjective technique that requires extensive experience to interpret the sensitivity of bacteria to bacteriophages. Its discriminatory power is often inadequate when it comes to finding the source of an infection, especially in countries where particular phage types are dominant; moreover, not-typable (NT) and reacts-but-does-not-conform (RDNC) isolates limit the suitability of phage typing for surveillance and outbreak detection, since not all isolates can be subtyped by this technique **(2-5)**. Nowadays, phage typing as a subtyping technique is often complemented with molecular methods like pulsed-field gel electrophoresis (PFGE). PFGE separates large DNA molecules, resulting from macrorestriction of the genome, by gel electrophoresis with alternately pulsed electrical fields in different directions. Differences in the observed electrophoretic patterns can be used to discriminate between isolates. PFGE is considered as the gold standard for subtyping of *Salmonella* **(6)**. Yet, recent studies suggest that multiple-locus variable-number of tandem repeats analysis (MLVA) improves surveillance, detection of outbreaks and of Salmonella infection sources, in particular for *S*. Typhimurium outbreaks **(2;4;7;8)**.

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MLVA targets rapidly evolving genomic elements known as tandem repeats. This allows them to be used in order to study genetic relatedness between isolates. The combination of the number of tandem repeats at a predefined number of MLVA loci results in a MLVA profile.

In Belgium, *S*. Typhimurium is, since 2006, the most frequently isolated serovar from humans, with an average of 1986 isolates reported per year (55.9% of all *Salmonella* isolates, average from 2006 up to 2012) **(9)**. Hence, subtyping of this serovar is very important for outbreak detection and tracing outbreak sources. The Belgian National Reference Centre for *Salmonella* and *Shigella* (NRCSS) relies on phage typing and antimicrobial susceptibility testing for routine surveillance of *S*. Typhimurium, complemented with PFGE during outbreak investigations. PFGE is a labour-intensive and time-consuming technique and, therefore, implementation of this subtyping method for routine surveillance is not feasible for the Belgian NRCSS. MLVA, which requires less hands-on time and allows for faster typing and easy inter-laboratory comparison of results, has been adopted by several European countries for surveillance and detection and investigation of outbreaks **(8)** and is currently also used by the Belgian NRCSS. Nonetheless, a recent study **(5)** on phage type, antimicrobial susceptibility and MLVA of a large collection of *S*. Typhimurium collected in Belgium during 2010-2012 demonstrated that, although the discriminatory power of MLVA allowed for an improvement of public health surveillance, phage typing was still necessary for isolates with a frequent MLVA profile to detect an outbreak and to uniquely characterise an outbreak isolate. Moreover, as some of the MLVA loci showed to be unstable, these genetic markers for subtyping should be interpreted with caution **(5)**. An ideal subtyping technique would combine different typing methods into a single molecular assay with stable markers. In this context, the PhD project SalMolType aims at developing a molecular subtyping technique, which should ideally be inexpensive, rapid, highly discriminative and robust and the used markers should be stable and testable in all isolates, so that a type can be assigned to all isolates **(6;10-12)**.
Until now, different kinds of DNA markers have been studied, alone or combined, for their suitability in a molecular subtyping scheme for *S*. Typhimurium. The number of markers included in the resulting methods and the level of multiplexing still feasible often determines the discriminatory power and rapidity of the method **(6)**.
In this study we discuss a proof-of-concept for a molecular subtyping method for *S*. Typhimurium that combines different types of markers, such as amplified fragment length polymorphisms (AFLP), prophage genomes, sequence repeats, antibiotic resistance markers and single-nucleotide polymorphisms (SNPs). To achieve a sufficient level of multiplexing in a high-throughput manner, we have implemented the Luminex® technology. This technology, which emerged in the 1990s, allows us to distinguish up to 500 different targets in one sample through its bead-based assays and offers possibilities in various fields of public health by enabling DNA, RNA and protein assays on a single instrument.

METHODS

Bacterial isolates

S. Typhimurium isolates were selected from the collection of the Belgian NRCSS. The selection includes 100 isolates, of which 13 isolates from two outbreaks, with different combinations of phage type, antimicrobial resistance pattern and MLVA profile. Besides the most frequently occurring phage types over the past years in Belgium, i.e. DT12, DT104L, DT120, DT193, DT195 and U302, also phage types DT1, DT35, DT104a, DT138, DT208, U311 and one RDNC isolate are included. In total, these isolates cover 25 different antimicrobial resistance patterns and 51 distinct MLVA profiles.

DNA isolation
DNA was isolated by boiling a single colony from an overnight culture on LB agar in water. After centrifugation, the supernatant was stored at -20°C and used for further analysis.

Stability experiment
Stability of selected markers was evaluated in 31 *S*. Typhimurium isolates of the most frequent phage types in Belgium. A series of 50 passages, starting from a single colony, was performed. Glycerol stocks were made before each fifth passage and DNA was isolated from cultures after the final passage **(10)**.

Marker selection
Markers were based on information found in literature. Markers that were considered informative through presence or absence were screened by PCR and gel electrophoresis on 30 isolates of the most common phage types in Belgium. Those markers which were not present or absent in all 30 isolates and thus have discriminatory power, were selected for the Luminex® assay.

Bead-based nucleic acid assay selection
The Luminex® technology, which is implemented through a MAGPIX® platform in the SalMolType project, utilises polystyrene beads with a different colour code for each set. Each bead set can be coated with a different capture probe. Multiplexing is then achieved by combining different bead sets to analyse one sample. During analysis, the set to which the bead belongs is determined by measuring the red fluorescence signal from the bead. Afterwards, the presence of a hybridised target oligonucleotide is detected by measuring the green fluorescence signal on that target. Each bead-based assay should thus incorporate a fluorophore in the target oligonucleotide. At this stage, different possibilities exist. At the start of the project, three bead-based DNA assay formats, *i.e.* direct hybridisation, allele specific primer extension (ASPE) and ligation dependent amplification (LDA), were compared with regard to usage possibilities, multiplexing capacity, workflow, optimisation and cost.

The direct hybridisation assay **(1)** starts with a multiplex PCR with labelled primers, after which the products are hybridised to beads, to which capture probes have been previously coupled. If primers labelled with biotin are used, an incubation step with streptavidin-phycoerythrin (SAPE) is necessary before analysis on a Luminex platform.
In contrast to the xMAP® technology that is used in direct hybridisation assays, where self-designed capture probes have to be attached to the beads, the xTAG® technology utilises beads with pre-coupled 24 bp anti-TAG-sequences. ASPE and LDA are built upon this technology and should integrate the complementary TAG-sequence in the target oligonucleotide.

ASPE **(2)** commences with a multiplex PCR, after which the products are treated with ExoSAP-IT™ to degrade remaining primers and dNTPs. Target-specific probes with a TAG-sequence at 5' are then annealed to the PCR product and extended with inclusion of biotin-dCTP. The ASPE products are analysed on a Luminex® platform after hybridisation to xTAG® beads and incubation with SAPE.
The first step in a LDA assay **(3) (figure 1)** is a multiplex ligation with a probe pair for each target. The upstream probe has a universal primer site, TAG-sequence and target-specific part; the adjacent downstream probe has a target-specific part and universal primer site. The second step comprises a singleplex PCR with universal primers, one of which is labelled. After hybridisation to xTAG® beads and an optional incubation with SAPE, the amplified ligation products are read out on a Luminex® platform.

Multiplex oligonucleotide design and in silico simulation of multiplex assays
For the design of probe pairs and primers for the multiplex liquid bead suspension array and in silico simulation of multiplex assays, two commercial software packages were compared, *i.e.* VisualOMPTM in combination with ThermoBLAST™ (DNA Software®) and PrimerPlex (PREMIER Biosoft).

RESULTS

Marker selection
Careful selection of markers is required to eliminate markers that would give the same result for all isolates tested in the assay and hence would not allow for discrimination between isolates.

After PCR screening of 30 isolates of phage types DT12, DT104L, DT120, DT193, DT195 and U302, 34 literature-based markers, detecting AFLP fragments, prophage genomes, sequence repeats, *Salmonella genomic island 1* (SGI1) and allantoin utilisation, were found to be informative.

Antibiotic resistance genes, SNPs, a marker detecting all *Salmonella* species and a marker specific to *S*. Typhimurium are also included in the current selection of markers.

Bead-based nucleic acid assay selection
To combine the selected markers in a multiplex liquid bead suspension array, three nucleic acid assay formats were considered. The advantages and disadvantages of the different bead-based nucleic acid assays are summarised in table 1. The LDA assay was chosen for development of a subtyping method for *S*. Typhimurium, since the multiplex step in this assay format is a ligation, which allows for a higher multiplex capacity and an addition of markers without redesign of the assay, in contrast to multiplex PCR. A LDA assay would thus be more modular than assays based on direct hybridisation or on ASPE. Additionally, the LDA does not require the use of neurotoxic tetramethylammonium chloride (TMAC) buffer, coupling of capture probes or optimisation of the hybridisation to Luminex® beads. Furthermore, the costs and total time to complete the assay were found to be acceptable.

Multiplex oligonucleotide design and in silico simulation of multiplex assays
Software that predicts melting temperatures, hybridisation structures and specificity for different types of oligonucleotides, and thereby simulating the multiplex assays *in silico*, can facilitate the design of multiplex assays by reducing the time and cost of experimentation. An extensive comparison was made between VisualOMP™ in combination with ThermoBLAST™ and PrimerPlex to identify the most suitable software package for multiplex oligonucleotide design and *in silico* simulation of multiplex assays. The comparison between both software packages is summarised in **table 2**.

For multiplex oligonucleotide design and *in silico* simulation of multiplex assays, VisualOMP™ in combination with ThermoBLAST™ was selected, despite of the annual licence fee, since it allows for the design of LDA probes, takes secondary structure folding into account and displays simulation results in easily interpretable graphics.

Proof-of-concept of a molecular LDA subtyping assay
Out of the 34 markers found informative through PCR screening, 10 markers with high discriminatory power—including AFLP, prophages, sequence repeats and SGI1—were selected for a proof-of-concept for the LDA subtyping assay. Two markers, one detecting all *Salmonella* species and the other specific to *S*. Typhimurium, were included as internal control for the DNA templates. Also included in the proof-of-concept are three markers for antibiotic resistance genes and one SNP.

The resulting 16-plex LDA was applied to 100 isolates and the 31 isolates after 50 passages in the stability experiment. The amplified ligation products were read out on a MAGPIX® platform. Each assay included a negative control (containing all reagents but no DNA template, for background signal measurement) and a positive control (containing all reagents and a mixture of DNA template representing all 16 markers) to verify the reaction. A clear separation was observed between negative and positive signals, measured as median fluorescence intensity (MFI).

Presence or absence of these 16 markers, as detected by the LDA, resulted in 20 distinct profiles in the 131 tested isolates. Inconsistencies between results of the PCR screening on 30 *S*. Typhimurium isolates and results of LDA were resolved by sequencing of the PCR product. The isolates in the stability experiment gave an identical profile before and after the 50 serial passages in LB medium.

Eleven LDA profiles were observed for a single isolate only. The eight DT1 isolates included in this study showed three different LDA profiles, which were not observed for other analysed isolates. Discrimination within isolates of the same phage type was also observed for isolates harbouring other phage types, including DT104. The LDA also allowed us to discriminate between isolates presenting the same MLVA profile. DT193 isolates presenting 2, 3 or 4 repeats at MLVA locus STTR9 showed distinct LDA profiles; the profile of the isolate with four repeats was not shared with other isolates. For DT12, different LDA profiles were also observed for isolates with 2, 3 or 5 repeats at STTR9. This shows that the discriminatory power of the proof-of-concept assay is already reasonable.

However, within clusters of isolates with the same LDA profile, seven clusters included two or more phage types and two or more MLVA profiles. The outbreak isolates showed the same LDA profile, but this LDA profile was also shared with other unrelated isolates, which have a different combination of phage type and MLVA profile than the outbreak isolates.

These findings necessitated extending the LDA assay with more informative markers to a higher level of multiplexing. Since the delivery of the proof-of-concept, the general experimental workflow has been used to include 37 additional literature-based markers in the subtyping assay so that the discriminatory power has considerably increased. Screening of a large collection of *S*. Typhimurium is currently ongoing, to evaluate the capabilities of this novel subtyping assay. Next-generation sequencing (NGS) of *S*. Typhimurium isolates and genome comparison is also ongoing, to increase the number of informative molecular markers for further fine tuning of the developed subtyping method.

DISCUSSION

Subtyping of foodborne pathogens is necessary for public health surveillance, outbreak detection and tracing of outbreak sources. Classically in surveillance, isolates of Typhimurium are phage typed and, often in case of an outbreak, further subtyped using molecular techniques such as PFGE or MLVA. Even if these techniques present undoubted additional value for subtyping, each of them has its intrinsic disadvantages. For this reason scientists are still searching for another subtyping method, which should ideally be inexpensive, rapid, highly discriminative and robust **(6;12)**.

Many of the proposed alternative molecular methods for subtyping of *S*. Typhimurium are limited by the number of molecular markers and by the capacity of multiplexing those markers in a single run. The Luminex® technology offers a multiplex capacity of 500 markers in a single run and has already been applied in different stages of *Salmonella* surveillance, but none of them fully meets the requirements of an optimal (sub)typing method. The commercial xMAP® *Salmonella* Serotyping Assay allows for characterisation of 90% of the most common Salmonella up to the serovar level, but is too expensive and interpretation of the results is too complex for routine use. At the subtyping level, *i.e.* below the serovar level, two assays have been published so far for *S*. Typhimurium, to our knowledge. The first assay uses two multiplex PCRs to analyse 30 prophage-related markers in a direct hybridisation assay **(13)**. Analysis of 438 *S*. Typhimurium isolates showed that the discriminatory power was not yet sufficient, since the assay assigned the same prophage profile to isolates with different phage types. The second Luminex® subtyping assay is the CRISPOL assay **(14)**, which stands for clustered regularly interspaced short palindromic repeats (CRISPR) polymorphisms. Here, a direct hybridisation assay is used for detection of 72 CRISPR spacers, after amplification of the spacer content of two CRISPR loci in a singleplex PCR. The CRISPOL assay was however not able to discriminate between *S*. Typhimurium isolates with phage type DT104, so that yet another subtyping method, *e.g.* MLVA, is needed for these isolates.

In the SalMolType project the LDA assay, which can achieve a high level of multiplexing by separating detection and amplification, was selected for development of a molecular alternative to traditional microbiological subtyping techniques. LDA is also cost-effective since the specific probe pairs are unlabelled. Numerous types of molecular markers can be combined and straightforwardly added or left out of the assay. New probe pairs are assigned a different TAG for detection on the Luminex® platform, while still using the universal primer pair for amplification of the signal, which makes the LDA a modular assay. This was particularly interesting in our case, as it was necessary to include additional informative markers to our proof-of-concept assay in order to increase its discriminatory power. Indeed, although discrimination within isolates of the same phage types was observed, even for isolates belonging to phage type DT104, which are difficult to type with PFGE **(15)**, different phage types and different MLVA profiles were observed in clusters showing the same LDA profile. Epidemiological concordance could also not be proven with the initial proof-of-concept assay, since the outbreak isolates did show the same LDA profile, but the LDA profile was shared with other, unrelated isolates. However, these results for the outbreak isolates were also observed for phage typing and for MLVA **(5)** and, as a consequence, the outbreak isolates could only be distinguished by the combination of phage type and MLVA profile. Extension of our proof-of-concept assay with other informative probe pairs, including these covering SNPs, should solve this issue. In fact, LDA is very specific due to severe constraints on the ligation reaction: the upstream probe must anneal adjacent to the downstream probe and a strict complementarity is necessary for the base pairs flanking the ligation site. The strict complementarity allows straightforward detection of SNPs.

An additional advantage of the Luminex® technology is that processing of the results generated by the LDA assay is facilitated by the single file output of the MAGPIX® platform, which can easily be handled without the need of expensive software. This will simplify the implementation of this assay in routine surveillance.

CONCLUSIONS

We have presented a proof-of-concept for the use of a LDA assay with analysis on a MAGPIX® platform for the subtyping of *S*. Typhimurium. More markers, based on literature and on NGS, have been included to increase discriminatory power, while optimisation of the workflow and of the assay has further reduced cost and time. This will lead to a cost-effective, rapid, highly discriminative, known target-based and robust method with stable markers, more efficiently addressing the needs of *S*. Typhimurium subtyping than MLVA and phage typing.

IMPACT ON PUBLIC HEALTH

Rapid and accurate subtyping of pathogens is important for surveillance and is crucial in determining the link between food and a human isolate and for the timely confinement of an outbreak. New molecular techniques offer an improvement in terms of standardisation, speed and accuracy with respect to conventional microbiological techniques. In the SalMolType project, an alternative method for subtyping of *S*. Typhimurium is developed, which is highly needed to tackle the disadvantages of the currently used subtyping methods.

Additionally, the project has a double multiplier effect for public health. On the one hand, it proposes a workflow for the development of a modular, multiplex molecular subtyping technique which can be implemented similarly for other pathogens monitored by the WIV-ISP. On the other hand, SalMolType also allowed for the introduction of the Luminex® technology in the WIV-ISP. The Luminex® technology paves the way to a broad range of proteomic and genomic applications, where multiplexing at both a high level and high-throughput, and with the same equipment, are within the range of possibilities. Researchers working on other ongoing research projects within the WIV-ISP are already developing Luminex® assays on the MAGPIX® platform, *e.g.* for detection of unauthorised genetically modified organisms (UGMs) in the food and feed chain (plants) in the UGMMONITOR project or for characterisation of shiga toxin-producing *Escherichia coli* in the IDESTEC project. Other applications of the Luminex® technology will be developed within the scope of the projects mycoMOLAIR (airborne fungi), epiURO (miRNA and DNA-methylation) and ORIENT-EXPRESS (orientation platform for pathogen and GMO).

Besides research projects in which a Luminex® assay is developed, the MAGPIX® platform is also already being used to test commercially available kits in the fields of clinical diagnostics and life science research: the xTAG® Respiratory Viral Panel (RVP) Fast allows for the detection of 16 RNA viruses and their subtypes from patients suspected of respiratory tract infections, while the Bio-Plex Pro™ human cytokine panel is a 15-plex immunoassay (sandwich ELISA) used to measure cytokine levels.
From the above it is clear that the Luminex® platform is a versatile instrument that both serves and incites public health research.

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FIGURE

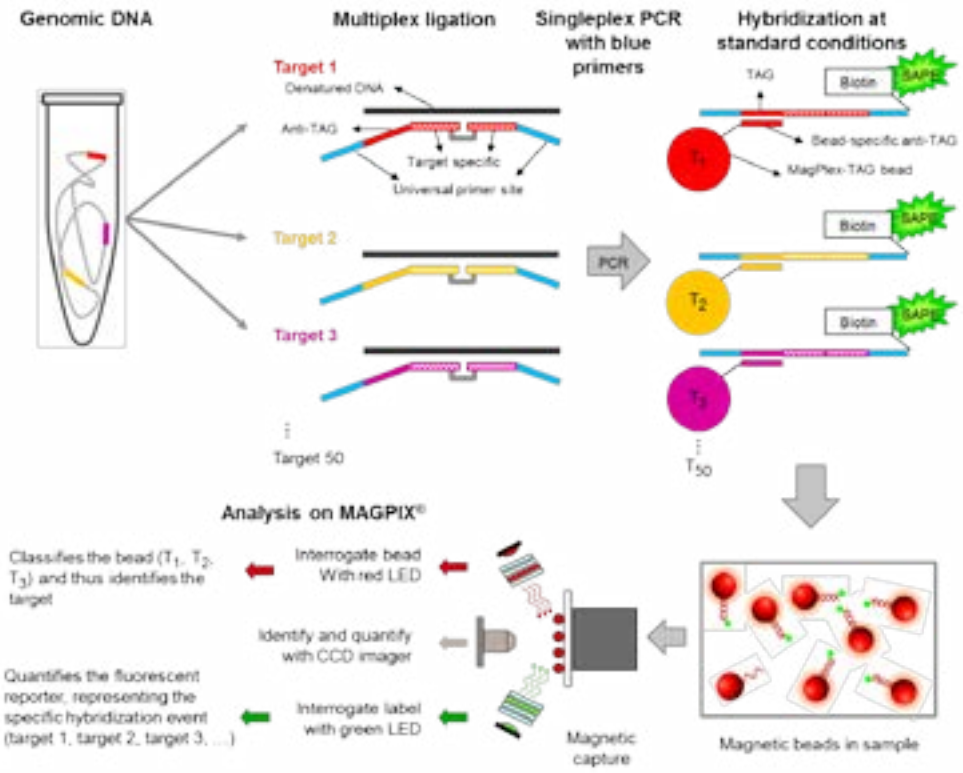

Figure 1- Multiplex ligation dependent amplification (LDA). Probes have a target-specific part (striped), a universal primer site (blue) and a TAG-sequence (red, yellow and purple). After a multiplex ligation reaction, a singleplex PCR is performed with universal primers, one of which is labelled with biotin. After hybridisation to xTAG® beads, a fluorescent label is introduced during an incubation with streptavidin-phycoerythrin (SAPE).

TABLES

Feature	Direct hybridisation	ASPE	LDA
Usage	Unrelated sequences, SNPs, multiple polymorphisms		
Detection of uncon-served sequences	- Less stringent	+ Stringent	+ Stringent
Multiplex step	- mPCR	- mPCR	+ Ligation
Addition of marker	- Redesign	- Redesign	+ No redesign
Buffers	- TMAC	+ Tm	+ Tm
Coupling of capture probe	- Yes	+ No	+ No
Hybridisation to beads	- Optimise: 45-55°C	+ Standard: 37°C	+ Standard: 37°C
PCR amplicon	- < 300 bp	+ all sizes	+ all sizes
Patent	+ No	+ No	- Yes
Cost	±	++	±
Total time	+ 3.5 hours	± 7 hours	± 6 hours

Table 1 - Overview of advantages (+) and disadvantages (-) of direct hybridisation, allele specific primer extension (ASPE) and ligation dependent amplification (LDA). mPCR: multiplex PCR; Tm: Tm buffer (0.1 M Tris-HCl pH 8, 0.2 M NaCl, 0.08% Triton® X-100); TMAC: tetramethylammonium chloride buffer (3 M TMAC, 0.1% Sarkosyl, 50 mM Tris, 4 mM EDTA).

Feature	VisualOMP™ + ThermoBLAST™	PrimerPlex
Company	DNA Software®	PREMIER Biosoft
Assay design	Direct hybridisation ASPE LDA	Direct hybridisation ASPE
Addition of TAG sequences	Copy-Paste	Automatically
Addition of existing sequences	Copy-Paste Fasta or Genbank file Entrez	Copy-Paste Fasta or Genbank file Entrez SNPdb
Addition of existing oligos	Copy-Paste Fasta or Genbank file	Copy-Paste Fasta or Genbank file
Modified oligos	Possible	Not possible
Specificity test	ThermoBLAST	BLAST
Secondary structure folding	Taken into account	Not included
Experiment conditions	Detailed	General
In silico simulation results	Detailed Melting curve Graphics	Detailed
Trial version	7 days Fully functional	30 days Restricted functionality
Help files	Extensive	Extensive
Tutorial	Extensive	Limited
Licence	Annual payment	One-time payment Annual maintenance fee

Feature	VisualOMP™ + Thermo-BLAST™	PrimerPlex
Company	DNA Software®	PREMIER Biosoft
Assay design	Direct hybridisation ASPE LDA	Direct hybridisation ASPE
Addition of TAG sequences	Copy-Paste	Automatically
Addition of existing sequences	Copy-Paste Fasta or Genbank file Entrez	Copy-Paste Fasta or Genbank file Entrez SNPdb
Addition of existing oligos	Copy-Paste Fasta or Genbank file	Copy-Paste Fasta or Genbank file
Modified oligos	Possible	Not possible
Specificity test	ThermoBLAST	BLAST
Secondary structure folding	Taken into account	Not included
Experiment conditions	Detailed	General
In silico simulation results	Detailed Melting curve Graphics	Detailed
Trial version	7 days Fully functional	30 days Restricted functionality
Help files	Extensive	Extensive
Tutorial	Extensive	Limited
Licence	Annual payment	One-time payment Annual maintenance fee

Table 2 - Comparison of software packages for multiplex oligonucleotide design and *in silico* simulation of multiplex assays. ASPE: allele specific primer extension; LDA: ligation dependent amplification.